REMARKS

Interview request

Applicants respectfully request a telephonic interview after the Examiner has reviewed the instant response and amendment. Applicants request the Examiner call Applicants' representative at 858 720 5133.

Status of the Claims

Pending claims

Claims 1, 4, 12, 16, 19, 20, 22 and 23 to 45 are pending. As claims 24 to 39 were withdrawn in the instant office action of December 30, 2004 ("OA"), claims 1, 4, 12, 16, 19, 20, 22, 23 and 40 to 45 are pending and under consideration.

Claims canceled, added and amended in the instant amendment

Claims 46 to 56 are added. Thus, after entry of the instant amendment, claims 1, 4, 12, 16, 19, 20, 22, 23 and 40 to 56, will be pending and under consideration.

Outstanding Rejections

The rejection of claims 1, 4, 12, 16, 19, 20, 22 and 23, is maintained, and claims 40 to 45 are newly rejected under 35 U.S.C. §112, first paragraph, enablement and written description requirements. The rejection of claims 1, 4, 12, 16, 19, 20, 22 and 23 is maintained, and claims 40 to 45 are newly rejected under 35 U.S.C. §112, second paragraph. The rejection of claims 1, 4, 12, 16, 19, 20, 22 and 23, is maintained, and claims 40 to 42 and 45 are newly rejected under 35 U.S.C. §112, first paragraph, as a new matter rejection.

Applicants respectfully traverse all outstanding objections to the specification and rejection of the claims.

Election and Restrictions

The Patent Office withdrew claims 24 to 39 (added in Applicants amendment of May 20, 2004), alleging that they are drawn to a non-elected invention and as lacking unity of invention. In

particular, it is alleged that the gene therapy of the newly added claims 24 to 39 lack unity of invention with claims drawn to protein therapy.

Applicants respectfully traverse and submit that the methods of independent claims 24, 25 and 31 to 33, and their dependent claims can be properly examined in the same application as methods using an exogenous dominant negative PLB protein functionally attached to a transport peptide or a vesicle-based transfer system, e.g., claims directed to treatments of heart failure comprising attenuating PLB-induced cardiac SR Ca^{2+} ATPase (SERCA2a) inhibition and enhancing contractility in a heart using a compound comprising an exogenous dominant negative PLB protein functionally attached to a transport peptide or a vesicle-based transfer system; or, methods for treatment of heart failure comprising enhancement of cardiac contractility by inhibition of PLB-sarcoplasmic reticulum calcium ATPase (SERCA2a) interaction.

Claim 24 is drawn to a method for attenuating PLB-induced cardiac SR Ca^{2+} ATPase (SERCA2a) inhibition in a heart cell or a muscle cell using an expression construct comprising a coding sequence for a dominant negative PLB functionally linked to a promoter active in the heart; claim 25 is drawn to a method for increasing cardiac SR Ca^{2+} ATPase (SERCA2a) activity in a heart cell or a muscle cell using an expression construct comprising a coding sequence for a dominant negative PLB functionally linked to a promoter active in the heart or the muscle cell; claim 31 is drawn to a method for enhancing contractility in a heart using an expression construct comprising a coding sequence for a dominant negative PLB functionally linked to a promoter active in the heart; claim 32 is drawn to a method for increasing heart activity using an expression construct comprising a coding sequence for a dominant negative PLB functionally linked to a promoter active in the heart, and the dominant negative PLB binds to wild-type PLB; claim 33 is drawn to a method for treating heart failure comprising using expression construct comprising a coding sequence for a dominant negative PLB functionally linked to a promoter active in the heart.

When making a lack of unity of invention requirement, the Office must explain why each group lacks unity with each other group, i.e., why there is no single general inventive concept, and specifically describe the unique special technical feature in each group. The Office alleges that

the new claims 24 to 39 lack unity of invention because, inter alia, they are drawn to protein administration versus nucleic acid administration, and that the technical feature of claims 24 to 39 is anticipated by Dillman, et al. (Sept. 16, 1998) Annals of the New York Academy of Sciences 853:43-48 (reference D1 in the international search report) ("Dillman") citing PCT Rule 13.2.

PCT Rule 13.2, "Circumstances in Which the Requirement of Unity of Invention Is To Be Considered Fulfilled," states "Where a group of inventions is claimed in one and the same international application, the requirement of unity of invention referred to in Rule 13.1 shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression 'special technical features' shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art."

Applicants respectfully submit that the methods of independent claims 24, 25 and 31 to 33, and their dependent claims can be properly examined under Rule 13.1 in the same application as methods using an exogenous dominant negative PLB protein functionally attached to a transport peptide or a vesicle-based transfer system. First, Applicants respectfully note that claims drawn to methods for the treatment of heart failure were grouped together in Group I (please see the restriction requirement dated February 13, 2003), which was subsequently elected in Applicant's response of March 12, 2003. In that response Applicants also elected mutant PLB, with traverse. In the office action of June 03, 2003, the Office rejoined species directed to wild type, mutant and truncated PLB.

Second, Applicants respectfully submit that Dillman is not prior art to the instant invention because, inter alia, it is an article describing Applicant's own work. Unless it is a statutory bar, a rejection based on a publication may be overcome by a showing that it was published either by applicant himself/herself or on his/her behalf. MPEP 715.01(c), page 700-243, 8th ed., Rev. 2, May 2004.

Thus, lastly, new claims 24 to 39 drawn to, inter alia, nucleic acid administration and claims drawn to, inter alia, protein administration share technical feature(s) that define their

contribution which each considered as a whole makes over the prior art. Each claim can be distinguished by, inter alia (can be distinguished over the prior art for reasons not limited to), providing a compound comprising an exogenous dominant negative PLB protein to attenuate PLB-induced cardiac SR Ca^{2+} ATPase (SERCA2a) inhibition in a heart cell or a muscle cell. Applicants note that no prior art has been cited regarding claims 1, 4, 12, 16, 19, 20, 22, 23 and 40 to 45, which are pending and under consideration, and respectfully submit that these and withdrawn claims 24 to 39, are novel and nonobvious over the prior art.

Support for the Claims

The specification sets forth an extensive description of the invention as set forth in the pending and amended claims. For example, support for claims directed to use of dominant negative PLB for increasing heart activity or treating heart failure can be found, inter alia, in the paragraph spanning pages 6 and 7, where the specification describes use of an exemplary dominant negative PLB that can bind to wild type PLB to imitate phosphorylation of PLB, i.e., to attenuate the inhibition of SERCA2a, or, and page 7, lines 2 to 8, where the specification describes use of an exemplary dominant negative PLB that can act as a competitive inhibitor of endogenous wild type PLB interactions with SERCA2a. Support for claims directed to methods for increasing heart activity or treating heart failure by administering a dominant negative PLB linked to a transport peptide, such as an antennapedia transport peptide, by a linker, e.g., polylysine, to a cardiomyocyte to enhance contractility of the cell can be found, inter alia, in Example 4, pages 28 to 29. Support for methods for increasing heart activity or treating heart failure wherein the antennapedia transport peptide comprises SEQ ID NO:7 or the exogenous dominant negative PLB protein comprises the first 16 residues of SEQ ID NO:8 can also be found, inter alia, in Example 4. Support for methods for increasing heart activity or treating heart failure using a composition where a PLB is functionally attached to a means for internalization of the compound through a cell membrane into a cytoplasm can be found, e.g., on page 23, lines 1 to 6. Support for methods for increasing heart activity or treating heart failure using a composition comprising a transport peptide having a receptor-independent transport mechanism can be found, inter alia, on page 23, line 26 to page 24, line 5. Support for methods for increasing heart activity or treating heart failure using a composition comprising a transport system comprising a mode of transfer comprising a transport or

a penetratin-based PLB peptide or including adenoviral or lipid vesicle based transfer, can be found, inter alia, on page 23, lines 1 to 13, of the specification. Support for methods for increasing heart activity or treating heart failure using a composition comprising a transport peptide selected from the group consisting of penetratin, adenovirus, bacterial and lipid vesicle based transport peptide, can be found, inter alia, in the originally filed claim 8. Support for methods for increasing heart activity or treating heart failure using a composition comprising a mutated exogenous dominant negative form of phospholamban (PLB) comprising a mutation at amino acid 16 from serine (S) to glutamic acid (E) (S16E) can be found, inter alia, in Example 5, on page 29, lines 27 to 30. Support for methods for increasing heart activity or treating heart failure using a composition comprising a mutated exogenous dominant negative form of phospholamban (PLB) comprising a mutation at amino acid residue 3 from lysine (K) to glutamic acid (E) and a mutation at amino acid residue 14 from arginine (R) to glutamic acid (E) (K3E/R14E), inter alia, on page 6, lines 27 to 29, and page 17, lines 12 to 16. Accordingly, no new matter is added by the instant amendment.

Information Disclosure Statements

Applicants thank the Examiner for expressly considering and initialing the Information Disclosure Statements (IDSs) and Forms PTO-1449, submitted September 23, 2003, and May 20, 2004.

Issues under 35 U.S.C. §112, first paragraph

Enablement

The rejection of claims 1, 4, 12, 16, 19, 20, 22 and 23, is maintained, and claims 40 to 45 are newly rejected under 35 U.S.C. §112, first paragraph, as allegedly not described in the specification in such a way as to enable one skilled in the art to which it pertains to make and/or use the invention (please see pages 3 to 5, of the OA).

The Patent Office states that the specification is enabling for gene-mediated ablation of endogenous PLB in a double-knockout mouse to enhance cardiac contractility (see page 3, lines 19 to 21, of the final office action of January 20, 2004), and gene therapy provides for a constant

expression of the drug inside the target cell (see page 3, lines 15 to 16, of the OA; see also, lines 8 to 10 of the office action of June 03, 2003).

However, the Office alleges that a question remains ... does the specification as filed teach the skilled artisan how to successfully administer the claimed dominant negative PLB-transport protein compositions for the treatment of heart disease. It is alleged that the specification does not provide reasonable enablement for administration of a composition of the invention (the dominant negative PLB functionally attached to a transport peptide or a vesicle-based transfer system) for the treatment of heart failure. Thus, the issue is not whether once inside a cell a dominant negative PLB-comprising composition of the invention can inhibit endogenous PLB activity to enhance cardiac contractility or treat heart failure, but, whether the specification enables one of skill how to apply or administer an exogenous PLB to successfully practice the methods of the invention, e.g., treat heart disease.

While Applicants aver that the specification does provide reasonable enablement to the skilled artisan, they first respectfully submit that the Patent Office has not met its initial burden to establish a reasonable basis to question the enablement provided for the claimed invention, and specifically address below how the art used to support the Office's enablement rejection is not sufficient to rebut the presumptively enabled specification.

In order to make a rejection, the Examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. In re Wright, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) (Examiner must provide a reasonable explanation as to why the scope of protection provided by a claim is not adequately enabled by the disclosure). A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. As stated by the court, "it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it

doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure." In re Marzocchi, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). See also MPEP §2164.04, 8th ed., rev. 2, May 2004, pg 2100-189.

The Patent Office cited the background section of applicants' specification to support its *prima facie* case of lack of enablement (no scientific literature was cited), see, e.g., lines 9 to 12, page 4, and lines 20 to 23, of page 5, of the office action of June 03, 2003. In particular, it was alleged that Applicants acknowledged (at page 5, line 7 to 10, background section) that the internalization of exogenous molecules to enhance cardiac contractility by live myocytes remains an unresolved issue. However, Applicants respectfully aver that the instant invention successfully addresses this issue by using a class of peptides or vesicles with translocating properties that have been demonstrated to carry hydrophilic compounds across a plasma membrane. The discussion in the same background information paragraph cited by the Office, reads in full (page 5, lines 7 to 29, of the instant specification):

> There is the understanding that interfering with the PLB-SERCA2a interaction may be a potential therapeutic target for the treatment of heart failure, however, the internalization of exogenous molecules to enhance cardiac contractility by live myocytes remains an unsolved issue. A means must be available to deliver any therapeutic agent directly to the cytoplasm and nucleus of cardiac myocytes. Penetratins, a class of peptides with translocating properties, have the ability to carry hydrophilic compounds across the plasma membrane. Research by Schwarze, et al. (Science 285:1569-1572; 1999) has demonstrated an approach to protein transduction using a penetratin-based fusion protein which contains an NH_2-terminal 11-amino acid protein transduction domain from the denatured HIV TAT protein (Genbank Accession No. AF033819). Using this non-cell-type specific transfer system allows direct targeting of oligopeptides and oligonucleotides to the cytoplasm and nucleus. One of the most well characterized translocation peptides is one that corresponds to residues 43 to 58 of antennapedia, a Drosophila transcription factor. It is believed that the translocation peptide interacts with charged phospholipids on the outer side of the cell membrane. Destabilization of the bilayer results in the formation of inverted micelles containing the peptide that travels across the cell membrane and eventually open on the cytoplasmic side. While the use of transport

> peptides to move cargo molecules into cells is not novel, it has not been demonstrated that transport peptides work well in cardiomyocytes.

Thus, contrary to the Office's allegations, Applicants have not acknowledged that the instant invention does not solve the problem of internalizing exogenous molecules into live myocytes. The comments in the background section only discussed the state of the art prior to the instant invention. In fact, in the specification Applicants expressly state that the invention provides a solution to this problem (see, e.g., page 6, lines 6 to 26). Accordingly, the Patent Office has not set forth a *prima facie* case of lack of enablement and the enablement rejection under section 112, first paragraph should be withdrawn.

It is also alleged that the specification is devoid of any teaching that this problem ("...the internalization of exogenous molecules to enhance cardiac contractility by live myocytes remains an unsolved issue") has been solved (see, e.g., line 11, page 4, of the office action of June 03, 2003). It is implied that the specification provides only a starting point for further research (see, e.g., line 14, page 4, of the OA). However, Applicants respectfully submit that the specification and the claimed invention clearly and expressly state the solution to this problem, for example, on page 7, lines 2 to 8, of the specification:

> In a third exemplary embodiment of the present invention, a compound consisting of a fusion of 1) a 16-residue transport peptide and 2) a truncated phospholamban protein or similar peptide are transported across the cell membranes in a receptor independent manner. Once inside the cytoplasm of the cardiomyocyte, the truncated phospholamban or similar peptide act as a competitive inhibitor of endogenous phospholamban interactions with SERCA2a.

Thus, the specification expressly describes one embodiment of the invention that solves the problem – a method wherein a truncated phospholamban protein or similar peptide are transported across the cell membranes in a receptor independent manner by virtue of its linkage to a transport peptide.

The specification further describes in detail exemplary peptide-based therapeutics for inhibition of PLB activity, inter alia, on pages 22 to 25 e.g.:

> Still further, the present invention provides for a peptide based therapeutic for the inhibition of phospholamban activity and a mode of

> delivery for such a therapeutic, based on the finding that PLB function can be inhibited in a dominant negative manner by overwhelming endogenous PLB with mutant PLB molecules, and that this inhibition leads to improved function in failing hearts.

(page 22, lines 25 to 31). The specification also describes an exemplary method for administering the compound of the invention comprising an exogenous dominant negative PLB functionally attached to a transport peptide on, inter alia, page 23, line 26 to page 24, line 5:

> The introduction of the molecule into the blood stream feeding the heart can is best achieved using a catheter located in the coronary artery. When the molecule is present in the extracellular environment surrounding a cardiomyocyte it rapidly enters the cardiomyocyte and inhibits the association of PLB with SERCA2a. The translocation function is attributable to the transport peptide which exhibits the ability to rapidly translocate itself and the attached "cargo" peptides across the cell membranes in a receptor independent manner. Once inside the cytoplasm of the cardiomyocyte, the PLB fragment will act as a competitive inhibitor of endogenous PLB interactions with SERCA2a.

In Example 4, pages 28 to 29, the specification describes the construction of an exemplary peptide complex of the invention, where a PLB inhibitor molecule was made by indirectly attaching a transport peptide and a PLB protein to a polylysine backbone. This peptide complex was tested and successfully demonstrated to translocate efficiently into live cardiomyocyte and be effective *in vivo*:

> The PLB inhibitor molecule was translocated efficiently into isolated neonatal rat cardiomyocytes and showed a resulting enhanced contractility of the cell, the results of which can be seen in FIGS. 5a and b. Myocytes that overexpressed the V49A PLB point mutation showed increased contractility, while myocytes which overexpressed the wild-type PLB exhibited decreased contractility when compared to non-infected myocytes.

Example 5, pages 31, describes cell-based studies to evaluate the ability of two penetratin-based peptides, two mutant PLB-penetratin peptides, and two multiple antigen peptides (MAP) to strengthen the contraction cycle of isolated mouse cardiomyocytes. Each of the penetratin-PLB peptides were evaluated to measure their ability to strengthen the contraction cycle of isolated mouse cardiomyocytes. Tests were repeated on several sets of cardiomyocytes to determine relative change in length through the contraction cycle with the TAT-PLB peptide (results set forth in Table 3 as samples 1-7) and without the peptide (controls 1-8). Each of the samples had an added

concentration of 10μM of the TAT-PLB peptide while the controls had no added peptide. Details of the test are further described on page 31, lines 1 to 8. It was concluded that while there appeared to be a trend towards a larger, faster contraction in the myocyte, T-test analysis not identify any statistical difference due to the high variability of the data.

This conclusion was cited by the Office as support that the specification does not reasonably enable the skilled artisan (see, e.g., "... despite the specifically articulated finding of Applicant in their own specification that there was no statistically significant effect on the heart in treated animals," page 3, lines 16 to 20 of the OA). However, Applicants respectfully submit that this single inconclusive experiment does not negate the fact that the specification as a whole reasonably described how to practice the invention to the skilled artisan. As set forth in Applicants' previous response (expressly incorporated herein), in a Rule 132 expert declaration co-inventor Dr. Chien declared that the specification presents data that demonstrates that PLB inhibitor molecule linked (using e.g., polylysine) to a transport peptide (e.g., antennapedia transport peptide) can induce enhanced contractility in a cardiac cell. For example, Example 4, pages 28 to 29, provides data that demonstrates that a mutant PLB molecule linked to a transport molecule via a polylysine was efficiently translocated into isolated rat cardiomyocytes. These cardiomyocytes showed enhanced contractility. The results of these experiments are illustrated in Figures 5a and 5b. Example 5, including Table 3, pages 20 to 31, describes experiments that administer a mutant PLB linked to a transport peptide to mouse cardiomyocytes. Table 3 summarizes data from those experiments. The Patent Office cited Example 5 to evidence its allegation of lack of enablement.

Furthermore, the specification describes (e.g., in Example 4) that an exemplary mutant PLB of the invention linked to a transport molecule via a polylysine can be translocated into rat cardiomyocytes, resulting in these cardiomyocytes showing enhanced contractility. As declared by Dr. Chien, the level of skill in this art at the time of the invention was very high. As declared by Dr. Chien, using the teaching of the specification, one skilled in the art could have selected routine screening protocols known in the art at the time of the invention to determine means to effectively apply and administer exogenous PLB as claimed. Dr. Chien declared that using the teaching of the specification, one of skill in the art could have determined protocols to apply and administer an

exogenous PLB protein linked to a transport protein to successfully practice the claimed methods of the invention.

The examiner must weigh all the evidence before him or her, including the specification and any new evidence supplied by applicant with the evidence and/or sound scientific reasoning previously presented in the rejection and decide whether the claimed invention is enabled. The examiner should never make the determination based on personal opinion. The determination should always be based on the weight of all the evidence. MPEP §2164.05, 8th edition, rev. 2, May 2004, pg 2100-190 to -191. Because the examiner must weigh all the evidence before him or her, the Office did not sufficiently consider and address Dr. Chien's previously submitted Rule 132 expert declaration regarding enablement, as discussed above, with reasons to doubt the objective truth of the statements contained therein. Applicants respectfully aver that their arguments with Dr. Chien's expert declaration are convincing to one skilled in the art and are sufficient to rebut any possible *prima facie* case of lack of enablement. Applicants have presented persuasive arguments that one skilled in the art would be able to make and use the claimed invention using the application as a guide. The evidence provided by applicant need not be conclusive but merely convincing to one skilled in the art. MPEP 2164.05, 8th edition, rev. 2, May 2004, pg 2100-190 to -191.

Additionally, for the record, Applicants respectfully note that use of peptides with translocating properties, e.g., the exemplary penetratin peptide of the invention, were and are recognized in the art as effective molecule carriers *in vivo*. For example, Rousselle (April 2000) "New Advances in the Transport of Doxorubicin through the Blood-Brain Barrier by a Peptide Vector-Mediated Strategy," Mol Pharmacol. 57:679-686 (Exhibit A attached), demonstrated the *in vivo* efficacy of penetratin-doxorubicin (dox) complexes using *in situ* rat brain perfusion technique and also by intravenous (i.v.) injection in mice. For example, i.v. administration of penetratin-complexed dox at a dose of 2.5 mg/kg in mice led to a significant increase in brain dox concentrations during the first 30 min of postadministration, compared with free dox.

Enablement is a legal determination of whether a patent enables one skilled in the art to make and use the claimed invention. Raytheon Co. v. Roper Corp., 724 F.2d 951, 960, 220 USPQ

592, 599 (Fed. Cir. 1983). Enablement is not precluded even if some experimentation is necessary, although the amount of experimentation needed must not be unduly extensive. Atlas Powder Co. v. E.I. Du Pont De Nemours & Co., 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir. 1984); W.L. Gore and Associates v. Garlock, Inc., 721 F.2d 1540, 1556, 220 USPQ 303, 315 (Fed. Cir. 1983). Whether large numbers of compositions (e.g., enzymes, antibodies, nucleic acids, and the like) must be screened to determine if one is within the scope of the claimed invention is irrelevant to an enablement inquiry. Experimentation is not considered undue, even if extensive, if it is routine or if the specification provides reasonable guidance regarding the direction of experimentation -- time and difficulty are not determinative of undue experimentation if the experimentation is routine. See PPG Indus., Inc. v. Guardian Indus. Corp., 75 F.3d 1558, 1564, 37 USPQ2d 1618, 1623 (Fed. Cir. 1996); In re Wands, 858 F.2d at 736-40, 8 USPQ2d at 1403-7; Hybritech, Inc. v. Monoclonal Antibodies, Inc.,802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), cert. denied, 480 U.S. 947 (1987) (acknowledging that, because practitioners in that art are prepared to screen large numbers of negatives in order to find a sample that has the desired properties, the screening that would be necessary to make additional antibody species was not "undue experimentation."). Thus, enablement is not precluded by the necessity to screen large numbers of compositions, as long as that screening is "routine," i.e., not "undue," to use the words of the Federal Circuit.

Analogously, practitioners of the medical sciences for the instant invention also recognized the need to screen numbers of negatives to find a sample that has the desired properties, for example, determining protocols to effectively apply and administer exogenous PLB protein as claimed. As declared by Dr. Chien (see his expert declaration in Applicants' last response), the screening procedures used to identify protocols to effectively apply and administer exogenous PLB protein were all well known in the art and at the time this application was filed. All were routine protocols for the skilled artisan. Thus, the skilled artisan using Applicants' written disclosure could practice the instant claimed invention without undue experimentation.

Accordingly, because the Patent Office has not set forth a *prima facie* case of lack of enablement, or, alternatively, because Applicants have submitted sufficient argument, including Dr. Chien's expert declaration, convincing to one skilled in the art that any possible *prima facie* case of

lack of enablement is rebutted, Applicants respectfully submit that the pending claims meet the enablement requirements under 35 U.S.C. §112, first paragraph. In light of the above remarks, Applicants respectfully submit that amended claims are fully enabled by and described in the specification to overcome the rejection based upon 35 U.S.C. §112, first paragraph.

Issues under 35 U.S.C. §112, first paragraph

Written Description

The rejection of claims 1, 4, 12, 16, 19, 20, 22 and 23, is maintained, and claims 40 to 45 are newly rejected under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter not described in the specification in such as way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention (please see pages 5 to 6, of the office action).

The term "dominant negative protein"

The Patent Office maintains the allegation that the exogenous dominant negative phospholambans (PLBs) recited in the claims are not described in the specification as filed (see page 8, of the office action of January 20, 2004, under "new rejections based on amendment").

Applicants respectfully submit that the claimed invention is sufficiently described in the specification so that one of ordinary skill in the art would be able to ascertain the scope of the claims with reasonable clarity and recognize that Applicants' were in possession of the claimed invention at the time of filing. In the January 20, 2004, office action, the Office notes that this issue is best resolved by Applicants pointing to the specification by page and line number where dominant negative PLBs are described. In reply, and in further response to their last response of May 20th, 2004 (incorporated herein), Applicants submit the specification expressly sets forth exemplary dominant negative PLBs of the invention on, inter alia, page 17, line 11 to 28:

> Using the knowledge that certain amino acid residues of PLB are required to maintain its inhibitory effects on SERCA2a, several single point mutations of PLB, V49A (Seq. ID. No. 2), E2A (Seq. ID. No. 3), R14E (Seq. ID. No. 4), S16N (Seq. ID. No. 5),a double point mutation of PLB, K3E/R14E (Seq. ID. No. 6) and a sense and antisense PLB (Seq. ID. No. 1) transgene can be engineered in order to disrupt the inhibitory effects of PLB on SERCA2a.

> Using recombinant adenoviruses for *in vivo* murine cardiac gene transfer, myocytes which overexpresses V49A, one of the single point mutations in PLB, exhibit an increase in contractility, while myocytes which overexpress the wild-type PLB exhibit a decrease in contractility when compared to non-infected myocytes, as is documented in FIG. 5. It can be concluded that the feasibility and utility of interfering with the interaction between the SERCA2a and PLB is clearly documented. The PLB-SERCA2a interaction appears to be the rate limiting step for establishing the set point of basal cardiac contractility and relaxation *in vivo*, and the disruption of this interaction can thereby short circuit the β-adrenergic pathway.

Thus, the specification expressly describes the structure of dominant negative PLBs of the invention. As noted in the passage, these mutants were designed using the knowledge that certain amino acid residues of PLB are required to maintain its inhibitory effects on SERCA2a, for which guidance is provided in the specification. For example, support for using a mutant PLB monomer that stabilizes a heteropentamer by imitating the effect of phosphorylation of wild type PLB monomer can be found, inter alia, on page 6, lines 27 to 30:

> In a second exemplary embodiment of the present invention, contractilin, a recombinant adenoviral mutant of PLB (K3E/R14E), binds to and imitates phosphorylation of phospholamban. This leads to an activation of the calcium pump of the sarcoplasmic reticulum thus increasing cardiac contractility.

The exemplary mutant PLB monomer K3E/R14E imitates the effect of phosphorylation by giving a protein a negative charge (and imitating the effect of phosphorylation) by replacing two uncharged amino acid residues, lysine (K) and arginine (R), with the negatively charged amino acid glutamic acid (E) at residues 3 and 14, respectively. Thus, clear structural guidelines are described for dominant negative PLBs used to practice the methods of the invention. As declared by Dr. Chien, determining additional dominant negative PLB species could have been determined by the skilled artisan using routine screening methods, including the exemplary methods described in the specification. Additionally, as noted by the cited passage, the feasibility and utility of interfering with the interaction between the SERCA2a and PLB was clearly documented in the specification.

The Federal Circuit has addressed the written description requirement in the context of biological sequences in Enzo Biochem. Inc. v. Gen-Probe Inc., 296 F.3d 1316, 63 USPQ2d 1609

(Fed. Cir. 2002). The Enzo court adopted the standard that "the written description requirement can be met by 'showing that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics . . . *i.e.*, complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.'" [Emphasis added] Id. at 1324, 63 USPQ2d at 1613. The court in Enzo adopted its standard from the USPTO's Written Description Examination Guidelines. See 296 F.3d at 1324, 63 USPQ2d at 1613 (citing the Guidelines). The Guidelines apply to proteins as well as DNAs. The Enzo court also stated:

> Similarly, in this court's most recent pronouncement, it noted:
>
> > More recently, in Enzo Biochem, we clarified that Eli Lilly did not hold that all functional descriptions of genetic material necessarily fail as a matter of law to meet the written description requirement; rather, the requirement may be satisfied if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure.

Amgen, 314 F.3d at 1332 [Amgen Inc. v. Hoechst Marion Roussel Inc., 314 F.3d 1313, 1330, 65 USPQ2d 1385, 1397 (Fed. Cir. 2003)]. Moba, B.V. v. Diamond Automation, Inc., 2003 U.S. App. LEXIS 6285; Fed. Cir. 01-1063, -1083, April 1, 2003.

Analogously, the structure of the genus of dominant negative PLBs used to practice the methods of the invention are sufficiently correlated to a particular, known structure (wild type PLB, and the exemplary dominant negative PLBs) and a physical (physico-chemical) property (adding charged amino acid residues to imitate the effect of phosphorylation, and the ability to bind, or not to bind, to cardiac SR Ca^{2+} ATPase (SERCA2a) or wild type PLB). Accordingly, the species of PLBs used to practice the methods of the invention are defined via shared physical and structural properties in terms that convey with reasonable clarity to those skilled in the art that Applicants, as of the filing date and at the time of the invention, were in possession of the claimed invention.

The claimed subject matter need not be described in haec verba to satisfy the description requirement. It is not necessary that the application describe the claim limitations exactly, but only so clearly that one having ordinary skill in the pertinent art would recognize from the disclosure that

applicants invented the claimed subject matter. In re Herschler, 591 F.2d 693, 700, 200 USPQ 711,717 (CCPA 1979). See also Purdue Pharma L.P. v. Faulding, Inc., 230 F.3d 1320, 1323, 56 USPQ2d 1481, 1483 (Fed. Cir. 2000) ("In order to satisfy the written description requirement, the disclosure as originally filed does not have to provide in haec verba support for the claimed subject matter at issue.").

Applicants respectfully aver that the specification describes the claim limitations sufficiently clearly that one having ordinary skill in the pertinent art would recognize from the disclosure that Applicants invented the claimed subject matter. The species of PLBs used to practice the methods of the invention are defined via shared physical and structural properties set forth in the specification in terms that convey with reasonable clarity to those skilled in the art that Applicants, as of the filing date and at the time of the invention, were in possession of the claimed invention. Accordingly, Applicants respectfully submit that the pending claims encompassing use of dominant negative PLBs meet the written description requirement under 35 U.S.C. §112, first paragraph.

The term "functionally linked"

The Office maintains the allegation that the term "functionally linked" recited in the claims are not described in the specification as filed (see page 5, line 22, to page 6, line 4, of the OA, and also page 8, of the office action of January 20, 2004, third paragraph of the "new rejections based on amendment" section).

The Office acknowledges that the specification teaches the subgenus of covalent linkages penetrating (transport) peptides to PLB by means of covalent peptide linkages. See claims 40 to 42.

However, it is alleged that because the generic term "functionally linked" (or "functionally attached", as claims 1 and 12 are directed to use of a compound comprising a PLB protein "functionally attached" to a transport peptide or a vesicle-based transfer system) encompasses any means of functionally attaching the recited members (transport peptides and PLB),

including non-covalent and chemical linkages, these additional functional linkages were not provided written description support in the specification as originally filed.

However, the specification clearly describes that the generic term "functionally linked" was intended to encompass more than covalent peptide linkages, e.g., on page 24, lines 13 to 18:

> While residues 43 to 58 of *Antennapedia* is a well characterized translocation peptide, and works well in the present invention, the present invention is not restricted to this method of transport. Other potential methods of transfer include the use of an 8-branched polylysine backbone to link the transport and cargo peptide, but it is not limited to this multi-branched structure. [emphasis added]

Furthermore, the specification expressly describes that alternative non-covalent linkages can be used to make the transport peptide-PLB linked compositions used in the methods of the invention (see page 28, lines 18 to 23, of the specification):

> A PLB inhibitor molecule was made by indirectly attaching a transport peptide and a PLB protein to a polylysine backbone. Alternatively, the PLB molecule could also have been made as a single long peptide consisting of a transport sequence tandemly attached to the cargo peptide sequence. [emphasis added]

The specification also expressly describes an exemplary chemical means (using a disulfide bond) of joining the transport peptide and the PLB (see page 29, lines 7 to 11, of the specification):

> Alternatively, the PLB inhibitor could have been constructed as a single peptide with the cargo and transport peptides attached to each other by a single peptide bond, or as the cargo and transport peptides attached to each other by a disulfide bond. [emphasis added]

Claim 8 as filed was directed to a peptide based therapeutic agent wherein the transport peptide is selected from the group consisting of penetratin, adenovirus, bacterial and lipid vesicle based transport peptide.

Thus, the specification expressly states that non-covalent and chemical linkages (e.g., adenovirus, bacterial and lipid vesicle based transport systems), in addition to covalent peptide linkages, are encompassed by the compositions used in the methods of the invention.

The claimed subject matter need not be described in haec verba to satisfy the description requirement. It is not necessary that the application describe the claim limitations exactly, but only so clearly that one having ordinary skill in the pertinent art would recognize from the disclosure that applicants invented the claimed subject matter. In re Herschler, 591 F.2d 693, 700, 200 USPQ 711,717 (CCPA 1979). See also Purdue Pharma L.P. v. Faulding, Inc., 230 F.3d 1320, 1323, 56 USPQ2d 1481, 1483 (Fed. Cir. 2000) ("In order to satisfy the written description requirement, the disclosure as originally filed does not have to provide in haec verba support for the claimed subject matter at issue.").

Applicants respectfully aver that the specification describes several alternative structural guidelines for "functional attachments" that can be used in making the PLB-comprising compositions of the invention. Thus, the specification sufficiently clearly describes these "functional attachments" such that that one having ordinary skill in the pertinent art would recognize from the disclosure that Applicants invented the claimed subject matter. The species of "functional attachments" used to practice the methods of the invention are structurally described and set forth in the specification in terms that convey with reasonable clarity to those skilled in the art that Applicants, as of the filing date and at the time of the invention, were in possession of the claimed invention. Accordingly, Applicants respectfully submit that the pending claims encompassing use of "functional attachments" or "functional linkages" using covalent peptides linkages, chemical linkages or non-covalent linkages meet the written description requirement under 35 U.S.C. §112, first paragraph.

Issues under 35 U.S.C. §112, second paragraph

The rejection of claims 1, 4, 12, 16, 19, 20, 22 and 23 is maintained, and claims 40 to 45 are newly rejected under 35 U.S.C. §112, second paragraph, for allegedly failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. In particular, the Office has maintained its objection to the term "dominant negative PLB", see, e.g., page 9 of the office action dated January 20, 2004.

In first addressing this issue, Applicants submitted an expert declaration by Dr. Chien (see response of May 20, 2004), who declared that the term "dominant negative" was well known in the art at the time of the invention and the specification uses the term as it would have been understood to one skilled in the art at the time of the invention. Dr. Chien declared that an example of a textbook definition for "dominant negative protein" now, and at the time of the invention (the definition has not changed) is "a mutant protein that as a result of the mutation has lost activity or function and interferes with the function of its corresponding wild-type protein." Applicants note that the specification, including the claims, use the term as it would have been understood to one skilled in the art at the time of the invention (and now, as the definition has not changed). Thus, at the time of the invention, and now, the skilled artisan would have understood that a "dominant negative protein" is a mutant protein that as a result of the mutation has lost activity or function and the mutant protein interferes with the function of its corresponding wild-type protein.

However, in the instant OA the Office is concerned, inter alia, that the term is not described in terms of a polypeptide by the phenotypic effect exerted by the administered polypeptide. The instant amendment addresses this concern.

The Office also requests a copy of an example of a textbook definition for "dominant negative protein" as discussed by Dr. Chien. To address this concern, a copy of a definition of this term as posted by the National Library of Medicine, NIH, is attached as Exhibit B. As declared by Dr. Chien, an expert in the relevant field, the term "dominant negative" was well known in the art at the time of the invention (and has the same meaning now) and the specification uses the term as it would have been understood to one skilled in the art at the time of the invention (and now).

Issues under 35 U.S.C. §112, first paragraph

Written Description – new matter

The rejection of claims 1, 4, 12, 16, 19, 20, 22 and 23, is maintained, and claims 40 to 42 and 45 are newly rejected under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the written description requirement. This is a new matter rejection (a new rejection based on amendment, see the OA, page 6, last paragraph).

Noting that in Applicants' last response the claims were amended to broadly recite the term "transport peptide", the Office alleges that because the term is interpreted to encompass any transport peptide it encompasses all transport mechanisms, including receptor-dependent and receptor-independent transport mechanisms. It is alleged that while receptor-independent transport peptides may be described by the specification, receptor-dependent transport mechanisms are not.

However, Applicants respectfully submit that the specification as filed described use of the broad term "transport peptide", see, e.g., page 6, lines 19 to 21:

> It is yet another advantage of the present invention to provide for a family of compounds that consist of a transport peptide covalently attached to wild-type, mutant, or truncated PLB. [emphasis added]

The specification also described use of the broad term "transport peptide" on page 24, lines 13 to 15:

> While residues 43 to 58 of Antennapedia is a well characterized translocation peptide, and works well in the present invention, the present invention is not restricted to this method of transport. Other potential methods of transfer include the use of an 8-branched polylysine backbone to link the transport and cargo peptide, but it is not limited to this multi-branched structure. [emphasis added]

The specification further expressly describes the invention as encompassing use of a broad range of cell targeting systems, see, e.g., page 23, lines 1 to 13, of the specification:

> For a therapeutic agent, such as an inhibitor of the PLB-SERCA2a interaction, to effect a target cell system, it must have a means for internalization through the cell membrane into the cytoplasm. The mode of transfer of the inhibitor can be by way of either a transport or penetratin based PLB peptide or it can also include adenoviral or lipid vesicle based transfer. For this purpose, a compound consisting of a fusion of a transport peptide and a PLB protein molecule is constructed. The transport peptide comprises a 16-residue from the sequence for antennapedia, a Drosophila transcription factor protein. The second peptide of the complex can be a truncated sequence of PLB protein. Further therapeutic benefits can be achieved using peptides that correspond to native PLB protein as well as a mutant or truncated form of PLB protein. [emphasis added]

Additionally, claim 8 as filed was directed to a peptide based therapeutic agent wherein the transport peptide is selected from the group consisting of penetratin, adenovirus, bacterial and lipid vesicle based transport peptide.

Thus, the specification describes the invention as encompassing a variety of cell targeting systems, e.g., where the mode of transfer of the PLB-comprising compound can be by way of either a transport or penetratin based PLB peptide (including penetratin, adenovirus, bacterial and lipid vesicle based transport peptides) or it can also include adenoviral or lipid vesicle based transfer. Please note that an adenoviral-based transport/ transfer system is a receptor-based (receptor-dependent) delivery system. Thus, the specification expressly described an exemplary composition of the invention comprising a receptor-based targeting system, or "transport peptide."

The invention encompasses any means for internalization of a PLB-comprising compound of the invention through a cell membrane into a cytoplasm, and several exemplary species are expressly described, including a 16-residue from the sequence for antennapedia.

The specification also expressly describes the invention as encompassing use of cell targeting systems that act in a receptor independent manner, see, e.g., page 23, line 26, to page 27, line 5, of the specification:

> The translocation function is attributable to the transport peptide which exhibits the ability to rapidly translocate itself and the attached "cargo" peptides across the cell membranes in a receptor independent manner. Once inside the cytoplasm of the cardiomyocyte, the PLB fragment will act as a competitive inhibitor of endogenous PLB interactions with SERCA2a. [emphasis added]

Accordingly, Applicants respectfully submit that the pending claims, which encompass all transport mechanisms, including receptor-dependent and receptor-independent transport mechanisms, meet the written description requirement under 35 U.S.C. §112, first paragraph.

CONCLUSION

In view of the foregoing amendment and remarks, Applicants respectfully aver that the Examiner can properly withdraw the rejection of the pending claims under 35 U.S.C. §112, first and second paragraphs. Applicants respectfully submit that all claims pending in this application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no. 220002066200. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

As noted above, Applicants have requested a telephone conference with the undersigned representative to expedite prosecution of this application. After the Examiner has reviewed the instant response and amendment, please telephone the undersigned at 858 7205133.

Dated: February 24, 2005

Respectfully submitted,

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New Advances in the Transport of Doxorubicin through the Blood-Brain Barrier by a Peptide Vector-Mediated Strategy

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Abstract

Many therapeutic drugs are excluded from entering the brain, due to their lack of transport through the blood-brain barrier (BBB). To overcome this problem, we have developed a novel method in which short, naturally derived peptides (16-18 amino acids) cross the cellular membranes of the BBB with high efficiency and without compromising its integrity. The antineoplastic agent doxorubicin (dox) was coupled covalently to two peptides, D-penetratin and SynB1. The ability of dox to cross the BBB was studied using an in situ rat brain perfusion technique and also by i.v. injection in mice. In the brain perfusion studies, we first confirmed the very low brain uptake of free radiolabeled dox because of the efflux activity of P-glycoprotein at the BBB. By contrast, we have demonstrated that when dox is coupled to either the D-penetratin or SynB1 vectors, its uptake was increased by a factor of 6, suggesting that the vectorized dox bypasses P-glycoprotein. Moreover, using a capillary depletion method, we have shown that vectorization of dox led to a 20-fold increase in the amount of dox transported into brain parenchyma. Intravenous administration of vectorized dox at a dose of 2.5 mg/kg in mice led to a significant increase in brain dox concentrations during the first 30 min of postadministration, compared with free dox. Additionally, vectorization led to a significant decrease of dox concentrations in the heart. In summary, our results establish that the two peptide vectors used in this study enhance the delivery of dox across the BBB.

Introduction

Drug delivery into the brain is often restricted by the blood-brain barrier (BBB), which regulates the exchange of substances between the peripheral circulation and the central nervous system. BBB acts first as an anatomical barrier because of the monolayer of endothelial cells, which are its main component. They exhibit specific properties such as the intercellular tight junctions, which prevent paracellular transport. More recently, the 170-kDa ATP-dependent efflux pump P-glycoprotein (P-gp), first described as participating in the multidrug resistance (MDR) mechanisms of tumor-cell drug resistance (Juliano and Ling, 1976) has been shown to be present at the luminal site of the endothelial cells of the BBB (Cordon-Cardo et al., 1989). As a result of the P-gp functional orientation (i.e., from brain to blood), P-gp may restrict the brain entrance or increase the brain clearance of a broad number of therapeutic compounds, including cytotoxic drugs (Gottesman and Pastan, 1993; Tsuji, 1998). As a consequence of P-gp expression at the BBB interface and overexpression at the tumoral cell level, the bioavailability of anticancer agents, which may act within the cellular compartment to treat brain tumors, is extremely low, which explains the failure of brain tumor chemotherapy (Blasberg and Groothuis, 1986). To overcome the limited access of drugs to the brain, different methods have been developed that achieve BBB uptake. Most of these methods are invasive and are characterized by intraventricular drug infusion or disruption of the BBB (Chamberlain et al., 1993; Kroll and Neuwelt, 1998). In the case of chemotherapeutic agents, few studies have explored the structural modification of drugs to bypass MDR (Klopman et al., 1997) or coadministration of the drug with P-gp modulators that inhibit the effect of P-gp at the BBB (Colombo et al., 1994; Drion et al., 1996; Hughes et al., 1998). Carrier-based approaches have also been developed. They consist, for example, of increasing drug delivery to the brain by the use of liposomes and nanoparticles (Huwyler et al., 1996; Mayer, 1998; Schroeder et al., 1998) or attachment of the drug to peptide-vectors transported into the brain by absorptive transcytosis through the BBB (see Pardridge, 1997, and references therein).

The pegelin and penetratin peptides (18 and 16 amino acids, respectively) translocate efficiently through biological membranes and have provided the basis for the development of new peptide-conjugated drugs for transport through BBB. Pegelin (such as SynB1) peptides are derived from natural peptides called protegrins (Harwig et al., 1995; Mangoni et al., 1996). They possess an amphipathic structure in which the positively charged and hydrophobic residues are separated in the sequence. They are thought to form an antiparallel β-sheet, constrained by two disulfide bridges (Aumelas et al., 1996). Replacement of the four cysteines with serines leads to linear peptides (pegelin) that are able to cross cell membranes efficiently without any cytolytic effect. The penetratin peptides are derived from the transcription factor antennapedia (Derossi et al., 1998). The region of the homeodomain of antennapedia responsible for internalization has been mapped to its third helix (Derossi et al., 1994). This finding has led to the demonstration that a 16-amino-acid peptide corresponding to the third helix translocates efficiently across biological membranes.

The aim of this study was to assess the efficacy of these peptides as vectors for delivery of drugs through the BBB. Doxorubicin (dox) was chosen as the vectorized drug because it is a widely used antineoplastic agent in the treatment of several cancers and has been shown to poorly cross the BBB and not to penetrate the brain tumor cells because of MDR mechanisms (Ohnishi et al., 1995; Mankhetkorn

et al., 1996). Various methods, such as the in situ brain perfusion technique (Takasato et al., 1984), have been used to evaluate brain uptake kinetics of drugs. We have applied this latter technique with some modifications (Smith, 1996). This method is simple and sensitive and allows the BBB to be exposed for a short time (15 to 90 s) to a drug under infusion conditions where the fluid composition and the rate of infusion are controlled. Complementary techniques were associated with it to measure the fraction of dox trapped into microvessel cells or present in brain parenchyma (Triguero et al., 1990). Finally, we investigated the overall bioavailability of the free and peptide-conjugated dox in mice. The results obtained in this study indicate that this approach could be used as a safe and effective delivery system for the transport of drugs across the BBB.

Materials and Methods

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Animals and Reagents

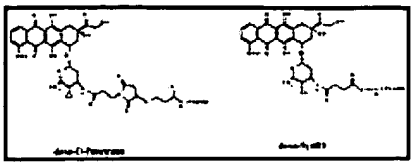
Male Sprague-Dawley rats (250-350 g; 8 weeks) were obtained from Iffa-Credo (L'Arbresle, France). Mice NMRI-nude (29 g; 7 weeks) were obtained from Janvier Breeding Center (Le Genest Saint Isle, France). Animals were maintained under standard conditions with ad libitum access to food and water. Rats were anesthetized with an i.p. injection of the combination ketamine hydrochloride (50 mg/ml; 70 mg/kg; Parke-Davis, Courbevoie, France) and diazepam (5 mg/ml; 7 mg/kg; Roche; Neuilly-Sur-Seine, France). Mice were anesthetized with isoflurane before sacrifice. The ethical rules of the French Ministry of Agriculture for experimentation with laboratory animals (law no. 87-848) were followed.

Preparation and Characterization of Peptide Conjugates

Peptide Synthesis. The peptides were assembled by conventional solid phase chemistry using a 9-fluorenylmethoxycarbonyl/tertiobutyl protection scheme (Atherton and Sheppard, 1989) and purified on preparative C18 reversed phase HPLC after trifluoroacetic acid (TFA) cleavage/deprotection. The lyophilized products were assessed by C18 reversed phase analytic HPLC. The peptide sequences were SynB1 (RGGRLSYSRRRFSTSTGR; molecular mass, 2099 D) and D-penetratin (rqikiwfqnrrmkwkk, the amino acids are in D form; molecular mass, 2245 D).

Dox-D-Penetratin Synthesis. Dox hydrochloride (1 molar equivalent; Fluka, Buchs, Switzerland) was suspended in dimethylformamide (DMF) containing diisopropylamine (2 molar equivalents; Fluka) (Fig. 1). *N*-hydroxysuccinimidylmaleimidopropionate (1 molar equivalent; Fluka) was added and incubated for 20 min. The thiol-containing peptide (either as a cysteine or as amino-terminal 3-mercaptopropionic acid solubilized in DMF) was then added to this reaction mixture, followed by a 20-min incubation. The acceptance criteria for the peptide and conjugates was HPLC purity of >98% at 215 and 480 nm in accordance with the molecular weight and fragmentation pattern for mass spectrometry. The molecular mass was found to be 3005 Da.

Fig. 1. Structure of dox-D-penetratin and dox-SynB1.



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Dox-SynB1 Synthesis. Dox hydrochloride was suspended in DMF containing diisopropylamine (Fig. 1). Succinic anhydride (1 molar equivalent; Fluka) dissolved in DMF was added and incubated for 20 min. The resulting dox hemisuccinate was then activated by addition of benzotriazol-1-yl-oxopyrrolidinephosphonium hexafluorophosphate (1.1 molar equivalents; Novabiochem) dissolved in DMF. The peptide was then added to the reaction mixture after 5 min of activation and left for another 20 min for coupling. Further processing and purity check of the conjugate was performed as described above. The molecular mass was 2723 Da.

Radiolabeling of Dox-D-Penetratin and Dox-SynB1. Preparations were performed as described above, except that [^{14}C]dox (55 mCi/mmol, 2.04 TBq/mol; Amersham, Les Ulis, France) was kept limiting by raising the stoichiometry of peptide, linkers, and activators to 1.3 eq in the coupling reactions. The specific activity of both compounds was (55 mCi/mmol, 2.04 Tbq/mol) and the molar ratio of dox/peptide was 1:1. The radiochemical purity was estimated to be >98% according to the 480-nm chromatograms.

Distribution Coefficient Determinations. The lipophilicity of the radiolabeled free and vectorized dox was estimated by measuring their partitioning between the perfusion buffer, pH 7.4, and 1-octanol. Distribution coefficients ($D_{octanol/buffer}$) were determined at volume ratios of 1:1 by vigorously shaking the two phases together. The samples were then incubated at 37°C for 30 min to facilitate phase separation. One sample of each phase was weighed and the radioactivity counted in a gamma counter. $D_{octanol/buffer}$ was calculated as: ([dpm/ml] in the octanol phase)/([dpm/ml] in the buffered saline phase). Experiments were done in triplicate and the mean of the log $D_{octanol/buffer}$ for dox, dox-D-penetratin, and dox-SynB1 were: 0.45 ± 0.06; -0.9 ± 0.08; and -1.44 ± 0.04, respectively.

Plasma Protein Binding Determination. Binding to rat plasma proteins of the radiolabeled free and vectorized dox was determined after incubation of each compound in rat plasma (Iffa Credo, L'arbresle, France) for 10 min at 37°C and ultrafiltration of the samples using the Centrifree Micropartition System (Amicon, Beverly, MA). Final concentrations in both phases were determined by counting the radioactivity as described above, and the bound fraction was calculated after three experiments. For dox, dox-D-penetratin, and dox-SynB1, mean values of bound fractions were: 87.66 ± 1.76, 99.49 ± 0.02, and 95.8 ± 0.38%, respectively.

In Situ Brain Perfusion

Blood to Brain Transfer of Dox. We used the in situ brain perfusion technique of Takasato et al. (1984) as described previously (Rousselle et al., 1998). The perfusion fluid was a bicarbonate-buffered physiological saline (128 mM NaCl, 24 mM $NaHCO_3$, 4.2 mM KCl, 2.4 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 0.22 mM $MgSO_4$, and 9 mM D-glucose, pH 7.4) infused at a flow rate of 10 ml/min. For some experiments, rat brains were perfused with plasma obtained the same day from heparinized donor rats at a flow rate of 5 ml/min, which is sufficient to perfuse the ipsilateral hemisphere at a reasonable pressure. [^{14}C]Dox (0.3 μCi/ml), [^{14}C]dox-D-penetratin (0.1 μCi/ml), and [^{14}C]dox-SynB1 (0.1 μCi/ml) were infused into the internal carotid artery for 60 s. [^{3}H]Sucrose (12.3 Ci/mmol; 1 μCi/ml; NEN, Paris, France) was used for each experiment as a marker of the BBB integrity. Some rats (n = 9) were also pretreated 5 min before perfusion with i.v. (±)-verapamil hydrochloride (1 mg/kg; Sigma, St. Quentin Fallavier, France) dissolved in 0.5 ml of 0.9% NaCl.

At the end of the perfusion, the rat was decapitated and the brain quickly removed. The right cerebral hemisphere was dissected on ice in six brain areas (frontal, occipital and parietal cortex, thalamus, hippocampus, and striatum). Brain regions and 50 μl of perfusion fluid were placed in preweighed scintillation vials and weighed. Brain and perfusion fluid samples were digested for 2 h in 1 ml of Soluene-350 (Packard, Rungis, France) at 60°C. Scintillation cocktail (10 ml; Picofluor, Packard) was added to each vial and the tracer contents were assessed by dual-label liquid scintillation counting program in a Tri-Carb model 1900TR liquid scintillation counter (Packard).

Dox uptake was expressed as a single-time-point, unidirectional transfer constant (K_{in}). Briefly, calculations were accomplished as described (Smith, 1996) from the relationship K_{in} = (Qtot − Vv · Cpf) / T · Cpf), where Qtot is the measured quantity of [^{14}C]dox in brain (vascular and extravascular) at the end of the experiment, Vv is the cerebral vascular volume, Cpf is the perfusion fluid concentration of [^{14}C]dox, and T is the perfusion time in seconds. Vv was evaluated by the sucrose space and calculated by the ratio between radioactivity of [^{3}H]sucrose (expressed in dpm of sucrose per gram of brain) and the perfusate sucrose concentration.

Washing Procedure. For this set of experiments, we used a dual-syringe infusion pump (Harvard Apparatus, Les Ulis, France) with one syringe containing the bicarbonate-buffered physiological saline with the radiotracer (syringe A) and the other without radiotracer (syringe B). The carotid catheter was connected to a four-way valve (Hamilton, Bonnaduz, Switzerland). After the carotid cannulation was completed and the appropriate connections were made, syringe A was discharged at a rate of 10 ml/min for 60 s. Syringe A was switched off and syringe B was switched on simultaneously to initiate the wash-out of the capillary space. After 30 s, the rat was decapitated. The transfer constant was measured using the equation K_{in} = Qtot / T · Cpf, where Qtot is the quantity of [^{14}C]dox in the extravascular brain.

Distribution in Brain Compartments. The distribution of [^{14}C]dox between brain microvascular and parenchymal compartments was assessed using the capillary depletion method of Triguero et al. (1990) with some modifications (Benrabh and Lefauconnier, 1996). Rats were perfused as described for the washing procedure. At the end of the wash-out, the right cerebral hemisphere was removed, cleaned of meninges and choroid plexus, weighed, and homogenized in 3.5 ml of capillary buffer (10 mM HEPES,

141 mM NaCl, 4 mM KCl, 1 mM NaH_2PO_4, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM D-glucose, pH 7.4) on ice. After 15 strokes, 4 ml of a chilled 40% neutral dextran solution was added to obtain a final concentration of 20%. All homogenizations were performed at 4°C in a very short time. After taking an aliquot of homogenate, the solution was centrifuged at 5400*g* for 15 min at 4°C in a swinging-bucket rotor. The pellet and supernatant were carefully separated and counted in the liquid scintillation counter. The pellet was composed mainly of brain capillaries and the supernatant reflected brain parenchyma.

Dox distribution in brain compartments was expressed as distribution volume (Vd; μl/g), defined as Vd = Qtis / Cpf, where Qtis is the measured quantity of [^{14}C]dox in brain compartments (total dpm per compartment/brain tissue weight) and Cpf is the perfusion fluid concentration (dpm/milliliters of perfusate).

Statistical Analysis. All experiments were performed on three to six rats. Data are expressed for individual cerebral areas or as the main value of the right cerebral hemisphere. Statistical comparisons conducted herein were accomplished by Student's test or ANOVA. Bonferroni's multiple comparison test was used post hoc only when ANOVA results were significant. Statistical difference was accepted at the $P < .05$ significance level. Data are presented as mean ± S.E.

Intravenous Administration in Mice

Dox and dox-SynB1 were i.v. injected in female Nude mice (via the tail vein) at a dose of 2.5 mg/kg (mg base of dox/kg; in 200 μl of NaCl 0.9%), which corresponded to 0.5 μCi per animal. At 1, 5, 15, 30, 60, 180, 480, and 1280 min after injection, animals (five animals per group) were anesthetized before sacrifice. Mice were sacrificed by cardiac puncture and blood samples were collected in glass tubes containing EDTA anticoagulant. Brain, heart, lungs, liver, and kidneys were removed for determination of total radioactivity. The plasma was recovered after centrifugation. The tissue samples were collected in scintillation tubes, immersed in liquid nitrogen, and stored at −20°C until analysis. The samples were fully used to quantify the radioactivity, and the radioactivity data was corrected in accordance with the quenching calculation. After radioactivity measurement, the results were transformed in micrograms of dox-equivalent per gram of plasma or tissue and represented as a mean ± S.E. of four to five animals.

Tissue to plasma partition coefficient (K_p) was determined by dividing the area under the average curve (AUC) calculated by the linear trapezoidal method for each time point between the tested tissue and plasma as $K_{p\ tn\rightarrow tn+1} = AUC_{\ tn\rightarrow tn+1}$ tissue / $AUC_{\ tn\rightarrow tn+1}$ plasma.

The term "tissue distribution advantage" (TDA) previously used by others (Malhotra et al., 1994) was introduced to evaluate the relative uptake behavior of dox-SynB1 versus free dox. TDA was calculated as the ratio of the respective tissue to plasma partition coefficients of the conjugated dox versus free dox at each time point according to TDA = $K_{p\ tn\rightarrow tn+1\ dox\text{-}SynB1} / K_{p\ tn\rightarrow tn+1\ dox}$. A TDA > 1 will define a specific tissue targeting of SynB1.

Stability In Vitro and In Vivo

Dox-SynB1 (1 ml) solution (2 mg/ml) was mixed with 4 ml of rat or mouse plasma (obtained from Iffa-Credo). At various times (0, 15, 25, 30, 40, 45, 60, 120, 180, and 210 min), 250-μl aliquots were withdrawn and quenched in 1 ml of acid mixture (H_2O/TFA 0.1%). The vectorized dox and metabolites were then extracted from plasma by applying the sample on a C18 solid phase extraction cartridge and eluting in 500 μl of acetonitrile/isopropanol/H_2O/TFA (50/20/30/5 ml) solution. The samples were then analyzed by HPLC on C18 column using acetonitrile/water gradient. The percentage of nondegraded vectorized dox and released dox was calculated.

In the in vivo stability study, mice were i.v. injected with dox-SynB1 at a dose of 2.5 mg/kg (milligram base of dox). The percentage of free released dox was measured by HPLC.

Results

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First, the tolerance of the BBB for the compounds used in this study was explored. [^{3}H]sucrose was used as a marker of brain vascular volume because it does not measurably penetrate the BBB during brief periods of perfusion. When 0.05 mg of either free or coupled dox were perfused, the vascular volumes were not significantly different among brain regions in all groups. They were about 10 μl/g of brain and of the same order of magnitude as those found in previous reports using the in situ brain perfusion method (Drion et al., 1996; Rousselle et al., 1998). This indicates that the permeability of the BBB was not changed. However, when 0.8 mg of dox-D-penetratin was perfused in rats, brain vascular volumes were 2-fold larger than those observed with other compounds or with 0.05 mg of dox-D-penetratin. Interestingly, the use of D-penetratin alone at the same concentration did not change the BBB permeability (data not shown), suggesting that alteration of the BBB may be caused by the complex dox-D-penetratin. We therefore used 0.05 mg of dox-D-penetratin or dox-SynB1 for the following brain perfusion experiments.

We then compared the brain uptake of free and coupled radiolabeled dox by measuring the total radioactivity in the brain after 60 s of brain perfusion. This perfusion time was chosen because it is short enough to limit the risks of drug metabolism or efflux from brain to blood but high enough to measure reasonable quantities of radiolabeled dox in brain tissues compared with the background noise of the detection method. Figure 2 shows that conjugation of dox with peptide vectors significantly enhances its brain uptake. An average of 6-fold increase in brain uptake was obtained for both dox-D-penetratin and dox-SynB1. To assess the brain distribution of these compounds, the brain was dissected into various areas: frontal, parietal, and occipital cortex, thalamus, hippocampus, and striatum. In rats perfused with dox, the brain uptake of this compound was very low and ranged from 0.18 ± 0.04 μl/s/g for the striatum to 0.78 ± 0.22 μl/s/g for the occipital cortex. Vectorization with either D-penetratin or SynB1 significantly increased the brain uptake of dox after 60 s of buffer perfusion in all six gray areas. The brain uptake of dox-SynB1 ranged from 1.6 ± 0.2 μl/s/g for the frontal cortex to 2.7 ± 0.4 μl/s/g for the parietal cortex. In the case of dox-D-penetratin, the brain uptake ranged from 1.4 ± 0.3 μl/s/g for the striatum to 3.6 ± 0.3 μl/s/g for the parietal cortex.

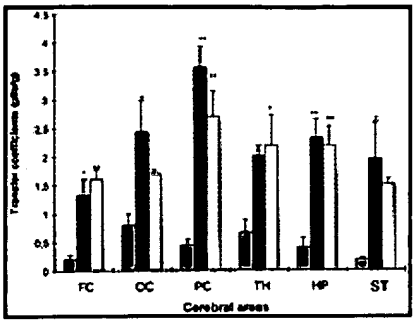

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Fig. 2. Transfer coefficients (K_{in}) for [^{14}C]dox, [^{14}C]dox-D-penetratin, and [^{14}C]dox-SynB1 uptake in six areas of rat brain after perfusion with buffer. Each bar represents a mean (± S.E.) for $n = 4$ animals. The animals were perfused for 60 s with 5.4 nmol/ml of dox (gray columns), 1.8 nmol/ml of dox-D-penetratin (filled columns), and 1.8 nmol/ml of dox-SynB1 (open columns). FC, OC, and PC, frontal, occipital and parietal cortex; TH, thalamus, HP, hippocampus; ST, striatum. $^{**}P < .01$; $^{*}P < .05$ versus free dox.

To evaluate whether free or coupled dox has actually crossed the BBB or is simply trapped within brain endothelial cells, two experiments were performed. In the first one, the brain was perfused for 60 s with radiolabeled compounds in physiological saline followed by a 30-s washing with tracer-free saline to remove tracer bound to the capillary luminal membrane. The total radioactivity measured was then compared with the one in animals that did not receive the wash-out procedure. After the washing procedure, the cerebral uptake of free and vectorized dox was significantly reduced, indicating that this procedure removed any [^{14}C]dox trapped within the microvessels or bound to the luminal membrane of its endothelium (Fig. 3). However, in rats perfused with either dox-D-penetratin or dox-SynB1, the brain uptake was still significantly increased (2.14 ± 0.23 and 1.50 ± 0.28 μl/s/g, respectively) compared with that of dox alone (0.25 ± 0.09 μl/s/g). In the second experiment, distribution in the brain capillary and parenchymal compartment was measured after perfusion and wash-out using the capillary depletion method of Triguero et al. (1990), which separates the whole brain into endothelial enriched (pellet) and depleted (supernatant) fractions. This procedure distinguishes between compounds remaining in the endothelial cells from those having crossed the abluminal endothelial membrane to enter the brain parenchyma. By this method, we have observed that about 50% of the dox-derived radioactivity was associated with the capillary, whereas less than 30% of the vectorized dox-derived radioactivity was in the endothelial cells after 60 s of perfusion followed by 30 s of wash-out (Fig. 4). In the parenchymal compartment, the ratio of vectorized dox versus free dox was about 20 for both peptide-vectors.

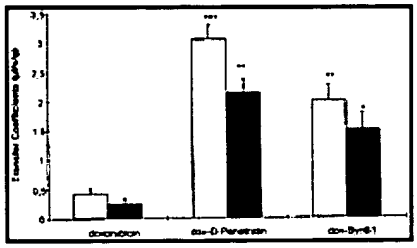

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Fig. 3. Transfer coefficients (K_{in}) for [^{14}C]dox, [^{14}C]dox-D-penetratin, and [^{14}C]dox-SynB1 uptake in right hemisphere of rat brain after 60-s perfusion with buffer and 30 s of wash-out. Values are mean ± S.E. ($n = 4$ rats). $^{***}P < .001$; $^{**}P < .01$; $^{*}P < .05$ versus free dox. Filled columns, wash-out; gray columns, no wash-out.

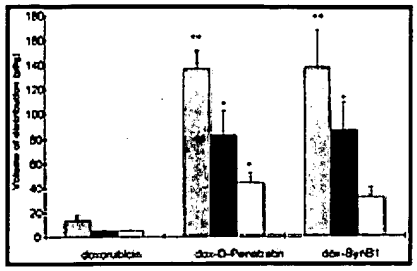

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Fig. 4. Distribution volumes of [^{14}C]dox, [^{14}C]dox-D-penetratin, and [^{14}C]dox-SynB1 in vascular pellet and supernatant fractions after wash-out after the capillary depletion method. Values are mean ± S.E. (n = 4 rats). $^{**}P < .01$; $^{*}P < .05$ versus free dox. Gray columns, homogenate; filled columns, parenchyma; open columns, endothelial cells.

To compare the vectorization of dox with the effect of a P-gp inhibitor, dox uptake was evaluated in rats pretreated with (±)-verapamil. This calcium-channel blocker is a P-gp inhibitor commonly used to reverse MDR in cell culture (Ford and Hait, 1990). Pretreatment with verapamil only slightly increased the cerebral uptake of dox after 60 s of perfusion and 30 s of wash-out (Table 1). However, this increase was not significant. Moreover, no change in brain uptake of vectorized dox was observed after pretreatment with verapamil.

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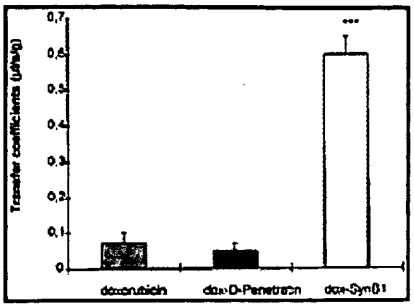
TABLE 1

Transfer coefficients for [^{14}C]dox, [^{14}C]dox-D-penetratin, and [^{14}C]dox-SynB1 uptake into rat brain. Data are presented as mean ± S.E.; n = 3-6. Perfusion time was 60 s and wash-out time was 30 s. Pretreatment with verapamil was carried out 5 min before perfusion at 1 mg/kg.

Finally, we investigated the effect of plasma protein binding on brain transfer of free and vectorized dox (Fig. 5). When the perfusion buffer was replaced by rat plasma, a dramatic decrease in dox-D-penetratin cerebral uptake was observed (0.05 versus 2.30 μl/s/g). The brain uptake of free dox was also significantly reduced (0.07 versus 0.44 μl/s/g) as it was for dox-SynB1 (0.60 versus 2.20 μl/s/g). Similar results were obtained after perfusion in the presence of 5% BSA in the saline buffer (data not shown). This is not surprising, because it has been shown previously that dox binds to plasma proteins and principally to albumin (Celio et al., 1982). Cerebral transfer coefficients of vectorized dox in plasma are also well correlated with the plasma protein binding measured in our study by ultrafiltration (87.8% for dox, 99.5% for dox-D-penetratin, and 95.8% for dox-SynB1). Consequently, we considered the possibility that the high protein binding of our peptide vectors may compromise dox delivery to the brain after peripheral administration.

Fig. 5. Transfer coefficients (K_{in}) for [^{14}C]dox, [^{14}C]dox-D-penetratin, and [^{14}C]dox-SynB1 uptake in rat brain after perfusion with plasma. Each bar represents a mean ± S.E. (n = 4 rats). Perfusion time was 60 s. $^{***}P < .001$ versus free dox.



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To check this last hypothesis and to confirm the ability to distribute more dox in the brain, we carried out in vivo experiments using i.v. injection. Free and SynB1-conjugated radiolabeled dox were injected into mice at a dose of 2.5 mg/kg (mg base of dox) via the tail vein. After different time points, the mice were sacrificed and the total radioactivity in plasma, brain, heart, lungs, kidneys, and liver was counted. After i.v. injection, the tissue and plasma distribution of dox-derived radioactivity were dramatically modified when the drug was conjugated to SynB1 (Fig. 6A). The plasma concentrations were higher for dox-SynB1 and decreased less rapidly than for the free dox. The brain distribution of dox was also apparently improved when the drug was conjugated to SynB1 (Fig. 6B). Interestingly, in the heart, where dox exerts its major toxicity, vectorization significantly reduced the dox concentrations (Fig. 6C). A similar decrease in accumulation of vectorized dox was observed in lungs. In kidneys and liver, a slight decrease in total radioactivity was observed for dox-SynB1 1 h after administration (data not shown). We also carried out a small-scale pilot experiment using D-penetratin as a vector and similar results as for SynB1 were obtained (data not shown).

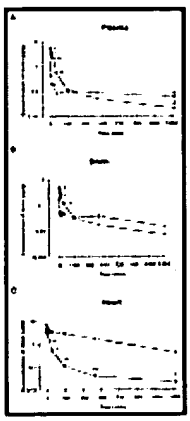

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Fig. 6. Plasma and tissues distribution after i.v. administration of free dox (◊) and dox-SynB1 (■) at a dose of 2.5 mg/kg. The concentrations are represented in microgram equivalents of dox on the basis of radioactivity measurements. The bars represent the S.E. of four to five animals. A, plasma; B, brain; C, heart. $*P < .05$; $**P < .01$ versus free dox.

To assess whether the modifications in tissue distribution observed with dox-SynB1 versus free dox

were caused only by an alteration of dox-SynB1 plasma pharmacokinetics, we calculated tissue-to-plasma-partition coefficients at each time point (Table 2) and compared them with those of dox alone. The calculated TDA was found to be >1 in brain during the first 30 min after administration, showing a more important brain uptake of dox-SynB1 than would have been expected from the observed increase of dox-SynB1 plasma levels (Fig. 7). In contrast, TDAs were <1 for heart, lungs, liver, and kidneys, showing a reduction in tissue exposure for these organs at all time points.

TABLE 2

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The area under plasma and tissues concentration curve (AUC) values were calculated for both dox-SynB1 and free dox from the time of injection to the given time point. The ratio of AUC tissue/AUC plasma of free and vectorized dox is represented here.

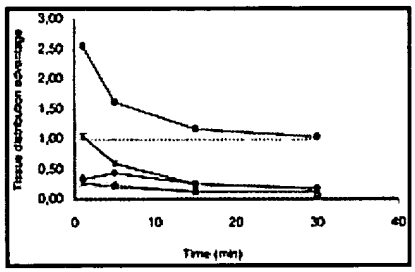

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Fig. 7. TDA was calculated as the ratio of the respective tissue to plasma partition coefficients of the vectorized dox versus free dox at each time point. ···, plasma; ¯, brain; △, heart; ×, lungs; ◇, liver; ●, kidneys.

To assess the stability and the fate of the dox-vector complex, we have carried out two preliminary experiments. In the first experiment, dox-SynB1 was incubated in rat and mouse plasma in vitro, and after various times, the fate and stability of the dox-SynB1 was analyzed by HPLC. Our results show that the conjugate has a degradation half-life of about 15 min in mice and rat plasma. The percentage of dox released was about 8% at 15 min. The rest of products corresponded mainly to degradation in the peptide. In the second experiment, dox-SynB1 was injected into mice and the percentage of released dox was measured by HPLC. In plasma, we found that about 3% of free dox was released from dox-SynB1 after 5 min postadministration (data not shown).

Discussion

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The discovery that synthetic peptides derived from natural peptides can be used successfully to deliver biologically active substances inside live cells (Derossi et al., 1998; Schwarze et al., 1999) has provided the basis for developing new effective strategies for drug delivery into the brain. For this reason we have coupled the anticancer drug dox to two different peptides: D-penetratin and SynB1, which were expected to increase the delivery of dox to rat brain.

We first evaluated the brain uptake of free and coupled dox after 60 s of in situ rat brain perfusion. Under these conditions, we only observed a low uptake of dox, comparable with values reported previously by Ohnishi et al. (1995) using the same method. However, this permeability is lower than would be expected based on the lipophilicity of the compound (log $D_{octanol/buffer}$ = 0.45). This low brain permeability could be explained by the efflux activity of P-gp at the BBB. Dox is actually transported by P-gp expressed at the brain capillaries in the physiological state (Ohnishi et al., 1995; Van Asperen et al., 1999) and could also be transported by the more recently characterized MDR-associated protein mrp1 (Abe et al., 1994). To overcome MDR mechanisms, dox was given in combination with P-gp inhibitor. However, if such drug combinations are effective in vitro, the high concentration of P-gp inhibitors necessary to overcome drug efflux limits their clinical application. Furthermore, coadministration of anticancer drugs and P-gp modulators may alter anticancer drug pharmacokinetics, leading to an exacerbation of anticancer drug toxicity (Krishna et al., 1997).

By coupling dox to D-penetratin and SynB1, we expected to increase its uptake in the brain and circumvent the efflux activity of P-gp. It is noteworthy that the coupling makes the dox less lipophilic ($logD_{octanol/buffer}$ = 0.45 for dox, −0.9 for dox-D-penetratin, and −1.44 for dox-SynB1), which in fact should reduce the permeability through the BBB. However, a significant increase in dox-derived radioactive brain uptake was obtained for the conjugated drug compared with free dox for all six gray areas studied. This increase in brain uptake obtained for both vectors might be explained by the translocation properties of these vectors and also by the fact that vectorized dox is not recognized by the P-gp. This is confirmed by pretreatment with verapamil, which did not change the brain uptake of the coupled dox, and only a slight increase was observed for free dox. To demonstrate that vectorized dox is not trapped inside the endothelial cells but actually crosses the BBB, we carried out the wash-out procedure and the capillary depletion method. Our results indicate that the amount of vectorized dox that was delivered to the brain parenchyma was about 20-fold higher than free dox, suggesting the efficiency of these peptide-vectors in delivering dox to the brain parenchyma. However, we observed a decrease in brain uptake (especially for dox-D-penetratin) when the cerebral perfusion was performed with plasma for a short period of time (60 s). Over a longer period of time, protein binding does not seem to hamper the brain distribution of vectorized dox as shown by the results obtained after i.v. administration in mice. These results are consistent with the hypothesis that the bound drug in plasma can dissociate from proteins and thus becomes available for brain transfer. When the permeability of the brain capillaries for the free drug is sufficiently high, a new equilibrium is rapidly achieved inside the capillaries leading to the release of some bound drug into a free form that then becomes available for brain transfer (Pardridge and Landaw, 1984; Jolliet-Riant and Tillement, 1999).

The pharmacokinetic profile of vectorized dox in plasma and tissues showed marked differences compared with free dox. In plasma, vectorization led to higher initial concentrations of dox-SynB1 than for free dox and the blood clearance of the vectorized dox was reduced during the first 180 min (area under the curve of dox-SynB1 was 5.51 times higher than for dox), allowing the compound to be more exposed to brain and other tissues. Assuming that dox-SynB1 is hydrolyzed in plasma with a stability half-life in plasma of about 15 min, this suggests that during at least 2 to 3 half-lives (i.e., 30 to 45 min) corresponding to the time window of the distribution phase, a higher tissue exposure was obtained for

dox-synB1 than for free dox.

Surprisingly, we found different distribution patterns of vectorized dox in tissues compared with free dox, suggesting a tissue-specific uptake of dox-SynB1. In fact, certain tissues like heart, lungs, and, to a lower extent, kidneys and liver, had a lower uptake of dox-SynB1 than free dox (TDA were in general <0.4). The lower accumulation in heart could be of great clinical interest, because the use of dox in chemotherapy has been hampered by its cardiotoxicity (Lefrak et al., 1973). The lower uptake observed for vectorized dox in lungs can also be regarded as an interesting property, because the lung is usually the first exposed organ after the i.v. route and is known to markedly distribute cationic molecules, causing toxicity (Bummer et al., 1995). Brain, rather than these tissues, seemed to accumulate vectorized dox. During the first 180 min after administration, the brain levels of dox-SynB1 were higher than those of free dox. Nevertheless, this might result from the increase in the systemic bioavailability of dox-SynB1. To verify this hypothesis, we calculated the brain distribution advantage, which shows that during the first 30 min after administration, brain uptake enhancement was higher than that observed in plasma. This observation confirms that during the period in which dox-SynB1 is not too much hydrolyzed, the more pronounced brain uptake results from the dox-SynB1 chemical entity interaction with endothelial cells of the BBB. This effect observed in vivo is well supported by the data from the in situ brain perfusion method, which showed a rapid transcytosis process across the BBB. For longer time-points, degraded forms of dox-SynB1 being predominant, no enhancement in dox brain uptake was observed. This suggests that enhancing the stability of the vectors might enhance the brain uptake of dox. A time balance between the kinetics of vector degradation and drug release in the targeted tissue has to be found by using less degradable amino acid sequences and appropriate linker. The challenge now is to develop peptide-vectors stable enough in plasma and a linker that will allow the drug to be cleaved off once it has crossed the BBB.

In summary, the tissue distribution shows two different organ patterns: tissues with less exposure (heart, lungs, liver, and kidneys) and tissues with higher exposure (brain). This clearly shows a tissue-specific uptake of the vectorized dox.

The mechanism by which these peptides cross the BBB is still under investigation. These peptides translocate efficiently across cell membranes and, at least in the case of D-penetratin, cell internalization does not seem to involve classical receptor-mediated endocytosis (Derossi et al., 1996). It is possible that once internalized, the peptides are addressed to a secretory compartment and re-exported into the brain parenchyma. Interestingly, Prochiantz and colleagues have demonstrated that homeoproteins —from which penetratin sequences were derived—can be secreted from live cells and gain access in vivo to a secretory compartment enriched in cholesterol and glycosphingolipids (Joliot et al., 1997, 1998).

These studies only test the feasibility of enhancing dox delivery to brain using peptide-vectors and do not address the pharmacodynamics of drug action in brain. It is crucial that the coupling of dox does not result in a loss of biological activity. Our preliminary experiments in cell culture using resistant cell lines show that the coupled dox with both D-penetratin and SynB1 bypasses P-gp and increases drug potency compared with free dox (unpublished observations). The next step will be to explore the antitumor

potential of vectorized dox in brain tumor models and new modified peptides.

In conclusion, this study demonstrates the successful application of the use of these peptide vectors for brain delivery of dox. A significant enhancement of dox uptake in brain was obtained after coupling dox with these peptides. Although these investigations focus on the delivery of dox, this approach should be applicable to other therapeutic drugs.

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Footnotes

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This article is dedicated to Prof. Alain Bonnet, who passed away in November 1999.

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Abbreviations

BBB, blood-brain barrier; P-gp, P-glycoprotein; dox, doxorubicin; MDR, multidrug resistance; TFA, trifluoroacetic acid; DMF, dimethylformamide; TDA, tissue distribution advantage.

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- Abe T, Hasegawa S, Taniguchi K, Yokomizo A, Kuwano T, Ono M, Mori T, Hori S, Kohno K and Kuwano M (1994) Possible involvement of multidrug-resistance-associated protein (MRP) gene expression in spontaneous drug resistance to vincristine, etoposide and adriamycin in human glioma cells. *Int J Cancer* **58:** 860-864[Medline].
- Atherton E and Sheppard RC (1989) *Solid Phase Peptide Synthesis: A Practical Approach.* IRL Press at Oxford University Press, Oxford, England.
- Aumelas A, Mangoni M, Roumestand C, Chiche L, Despaux E, Grassy G, Calas B and Chavanieu A (1996) Synthesis and solution structure of the antimicrobial peptide protegrin- 1. *Eur J Biochem* **237:** 575-583[Abstract].
- Benrabh H and Lefauconnier JM (1996) Blood-endothelial cell and blood-brain transport of L-proline, alpha-aminoisobutyric acid, and L-alanine. *Neurochem Res* **21:** 1227-1235[Medline].
- Blasberg RG and Groothuis DR (1986) Chemotherapy of brain tumors: Physiological and pharmacokinetic considerations. *Semin Oncol* **13:** 70-82[Medline].
- Bummer PM, Aziz S and Gillepsie MN (1995) Inhibition of pulmonary surfactant biophysical

activity by cationic polyamino acids. *Pharm Res* **12:** 1658-1663[Medline].
- Celio LA, Digregorio GJ, Ruch E, Pace JN and Piraino AJ (1982) Doxorubicin concentrations in rat plasma, parotid saliva, and bile and protein binding in rat plasma. *Arch Int Pharmacodyn Ther* **260:** 180-188[Medline].
- Chamberlain MC, Khatibi S, Kim JC, Howell SB, Chatelut E and Kim S (1993) Treatment of leptomeningeal metastasis with intraventricular administration of depot cytarabine (DTC 101). A phase I study. *Arch Neurol* **50:** 261-264[Abstract].
- Colombo T, Zucchetti M and D'Incalci M (1994) Cyclosporin A markedly changes the distribution of doxorubicin in mice and rats. *J Pharmacol Exp Ther* **269:** 22-27[Abstract].
- Cordon-Cardo C, O'Brien JP, Casals D, Rittman-Grauer L, Biedler JL, Melamed MR and Bertino JR (1989) Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc Natl Acad Sci USA* **86:** 695-698[Abstract].
- Derossi D, Calvet S, Trembleau A, Brunissen A, Chassaing G and Prochiantz A (1996) Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. *J Biol Chem* **271:** 18188-18193[Abstract/Free Full Text].
- Derossi D, Chassaing G and Prochiantz A (1998) Trojan peptides: The penetratin system for intracellular delivery. *Trends Cell Biol* **8:** 84-87.[Medline]
- Derossi D, Joliot AH, Chassaing G and Prochiantz A (1994) The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem* **269:** 10444-10450 [Abstract/Free Full Text].
- Drion N, Lemaire M, Lefauconnier JM and Scherrmann JM (1996) Role of P-glycoprotein in the blood-brain transport of colchicine and vinblastine. *J Neurochem* **67:** 1688-1693[Medline].
- Ford JM and Hait WN (1990) Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* **42:** 155-199[Medline].
- Gottesman MM and Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62:** 385-427[Medline].
- Harwig SS, Swiderek KM, Lee TD and Lehrer RI (1995) Determination of disulphide bridges in PG-2, an antimicrobial peptide from porcine leukocytes. *J Peptide Sci* **1:** 207-215.[Medline]
- Hughes CS, Vaden SL, Manaugh CA, Price GS and Hudson LC (1998) Modulation of doxorubicin concentration by cyclosporin A in brain and testicular barrier tissues expressing P-glycoprotein in rats. *J Neurooncol* **37:** 45-54[Medline].
- Huwyler J, Wu D and Pardridge WM (1996) Brain drug delivery of small molecules using immunoliposomes. *Proc Natl Acad Sci USA* **93:** 14164-14169[Abstract/Free Full Text].
- Joliot A, Maizel A, Rosenberg D, Trembleau A, Dupas S, Volovitch M and Prochiantz A (1998) Identification of a signal sequence necessary for the unconventional secretion of Engrailed homeoprotein. *Curr Biol* **8:** 856-863[Medline].
- Joliot A, Trembleau A, Raposo G, Calvet S, Volovitch M and Prochiantz A (1997) Association of Engrailed homeoproteins with vesicles presenting caveolae-like properties. *Development* **124:** 1865-1875[Abstract/Free Full Text].
- Jolliet-Riant P and Tillement JP (1999) Drug transfer across the blood-brain barrier and improvement of brain delivery. *Fundam Clin Pharmacol* **13:** 16-26[Medline].
- Juliano RL and Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* **455:** 152-162[Medline].
- Klopman G, Shi LM and Ramu A (1997) Quantitative structure-activity relationship of multidrug resistance reversal agents. *Mol Pharmacol* **52:** 323-334[Abstract/Free Full Text].
- Krishna R, De Jong G and Mayer LD (1997) Pulse exposure of SDZ PSC 833 to multidrug resistant P388/ADR and MCF7/ADR cells in the absence of anticancer drugs can fully restore sensitivity to doxorubicin. *Anticancer Res* **17:** 3329-3334[Medline].
- Kroll RA and Neuwelt EA (1998) Outwitting the blood-brain barrier for therapeutic purposes: Osmotic opening and other means. *Neurosurgery* **42:** 1083-1099[Medline].
- Lefrak EA, Pitha J, Rosenheim S and Gottlieb JA (1973) A clinicopathologic analysis of

adriamycin cardiotoxicity. *Cancer* **32:** 302-314[Medline].
- Malhotra BK, Lemaire M and Sawchuk J (1994) Investigation of the distribution of eab 515 to cortical ECF and CSF in freely moving rats utilizing microdialysis. *Pharmacol Res* **11:** 1223-1232.
- Mangoni ME, Aumelas A, Charnet P, Roumestand C, Chiche L, Despaux E, Grassy G, Calas B and Chavanieu A (1996) Change in membrane permeability induced by protegrin 1: Implication of disulphide bridges for pore formation. *FEBS Lett* **383:** 93-98[Medline].
- Mankhetkorn S, Dubru F, Hesschenbrouck J, Fiallo M and Garnier-Suillerot A (1996) Relation among the resistance factor, kinetics of uptake, and kinetics of the P-glycoprotein-mediated efflux of doxorubicin, daunorubicin, 8-(*S*)-fluoroidarubicin, and idarubicin in multidrug-resistant K562 cells. *Mol Pharmacol* **49:** 532-539[Abstract].
- Mayer LD (1998) Future developments in the selectivity of anticancer agents: Drug delivery and molecular target strategies. *Cancer Metastasis Rev* **17:** 211-218[Medline].
- Ohnishi T, Tamai I, Sakanaka K, Sakata A, Yamashima T, Yamashita J and Tsuji A (1995) In vivo and in vitro evidence for ATP-dependency of P-glycoprotein- mediated efflux of doxorubicin at the blood-brain barrier. *Biochem Pharmacol* **49:** 1541-1544[Medline].
- Pardridge WM (1997) Drug delivery to the brain. *J Cereb Blood Flow Metab* **17:** 713-731 [Medline].
- Pardridge WM and Landaw EM (1984) Tracer kinetic model of blood-brain barrier transport of plasma protein- bound ligands. Empiric testing of the free hormone hypothesis. *J Clin Invest* **74:** 745-752[Medline].
- Rousselle CH, Lefauconnier JM and Allen DD (1998) Evaluation of anesthetic effects on parameters for the in situ rat brain perfusion technique. *Neurosci Lett* **257:** 139-142[Medline].
- Schroeder U, Sommerfeld P, Ulrich S and Sabel BA (1998) Nanoparticle technology for delivery of drugs across the blood-brain barrier. *J Pharmacol Sci* **87:** 1305-1307[Medline].
- Schwarze SR, Ho A, Vocero-Akbani A and Dowdy SF (1999) In vivo protein transduction: Delivery of a biologically active protein into mouse. *Science (Wash DC)* **285:** 1569-1572 [Abstract/Free Full Text].
- Smith QR (1996) Brain perfusion systems for studies of drug uptake and metabolism in the central nervous system. *Pharm Biotechnol* **8:** 285-307[Medline].
- Takasato Y, Rapoport SI and Smith QR (1984) An in situ brain perfusion technique to study cerebrovascular transport in the rat. *Am J Physiol* **247:** H484-H493[Abstract/Free Full Text].
- Triguero D, Buciak J and Pardridge WM (1990) Capillary depletion method for quantification of blood-brain barrier transport of circulating peptides and plasma proteins. *J Neurochem* **54:** 1882-1888[Medline].
- Tsuji A (1998) P-glycoprotein-mediated efflux transport of anticancer drugs at the blood-brain barrier. *Ther Drug Monit* **20:** 588-590[Medline].
- Van Asperen J, Van Tellingen O, Tijssen F, Schinkel AH and Beijnen JH (1999) Increased accumulation of doxorubicin and doxorubicinol in cardiac tissue of mice lacking mdr1a P-glycoprotein. *Br J Cancer* **79:** 108-113[Medline].

0026-895X/00/040679-08$3.00/0
MOLECULAR PHARMACOLOGY, 57:679-686 (2000).

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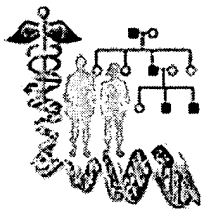

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Dominant negative mutation

Definition(s)

- A mutation whose gene product adversely affects the normal, wild-type gene product within the same cell, usually by dimerizing (combining) with it. In cases of polymeric molecules, such as collagen, dominant negative mutations are often more deleterious than mutations causing the production of no gene product (null mutations or null alleles).

  Definition from: GeneTests ⇨ from the University of Washington and Children's Health System, Seattle

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